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(71) Applicant *(for all designated States except US)*: **EMORY UNIVERSITY** [US/US]; 1784 North Decatur Road, Atlanta, GA 30322 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CHAIKOF, Elliot, L.** [US/US]; 150 Wicksford Glen, Atlanta, GA 30350 (US). **CAZALIS, Chrystelle, S.** [FR/FR]; Ingenieur de Recherche Flamel, Technologies Parc Club due Moulin a Vent, 33, Av. du Dr. Geoges Levy, FR-69693, Venissieux Cedex (FR). **HALLER, Carolyn, A.** [US/US]; 6851 Roswell Road, NE, Apt. #H28, Atlanta, GA 30328 (US).

(74) Agents: **PENNER, Steven, J.** et al.; Greenlee, Winner and Sullivan, P.C., 4875 Pearl East Circle, Suite 200, Boulder, CO 80301 (US).

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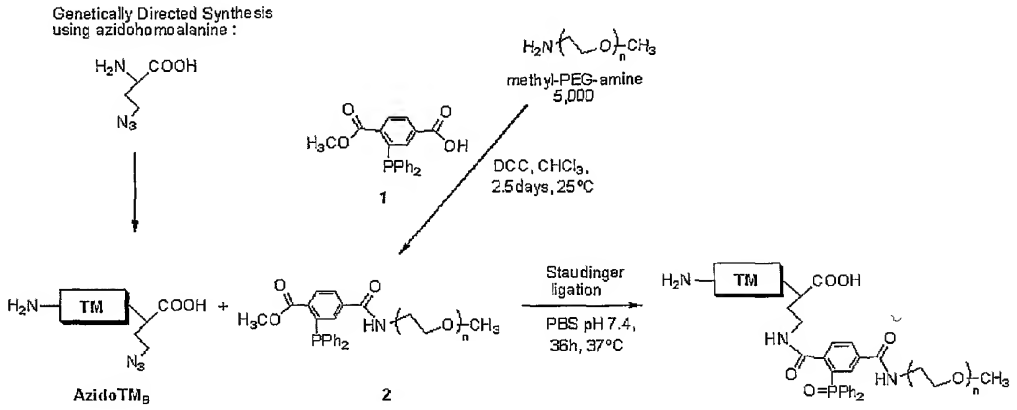

Scheme A

(57) Abstract: The transmembrane human protein thrombomodulin (TM), as a critical regulator of the protein C pathway, represents the major anticoagulant mechanism that is operative in both normal and injured blood vessels under physiologic conditions in vivo. Compositions and methods are disclosed relating to thrombomodulin derivatives and conjugates, including methods for site-specific pegylation and compositions of a truncated thrombomodulin derivative.

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THROMBOMODULIN DERIVATIVES AND CONJUGATES

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit of U. S. Provisional Application No. 60/546,436 by Chaikof et al. filed February 20, 2004, which is incorporated herein by reference in entirety.

STATEMENT ON FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made, at least in part, with government support under Grant No. NIH RO1HL56819 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] The transmembrane human protein thrombomodulin (TM), as a critical regulator of the protein C pathway, represents the major anticoagulant mechanism that is operative in both normal and injured blood vessels under physiologic conditions in vivo. An effective blood-contacting surface is dependent upon the presence of physiologically relevant antithrombogenic mechanisms that are incorporated into an engineered blood-material interface. Full length native human TM can be incorporated into membrane-mimetic films or surfaces by fusion/adsorption processes; a major drawback of these materials, however, is a loss of TM stability and/or functional activity with time. Moreover, in existing protocols for covalent immobilization of TM onto polymeric surfaces, the protein immobilization procedure involves freely available amino or carboxyl functionalities of TM, some of which may be within or near a bioactive site. The use of such functionalities can significantly reduce the functional bioactivity of TM after surface coupling. There is therefore a need for compositions and methods in this field to serve as effective alternatives.

SUMMARY OF THE INVENTION

[0004] In general the terms and phrases used herein have their art-recognized meaning, which can be found by reference to standard texts, journal references and contexts known to those skilled in the art. The following definitions are provided to clarify their specific use in the context of the invention.

[0005] When used herein, "thrombomodulin" refers to a protein molecule that is capable of involvement in the conversion of protein C to the activated protein C (protein Ca). In a particular example, the molecule relates to a specific endothelial cell receptor that forms a 1:1 stoichiometric complex with thrombin. In a preferred embodiment, the molecule is human and is also known as fetomodulin and CD141 antigen. In a particular embodiment, the term refers to a native molecule with nucleic acid or protein sequence information corresponding to that of accession number NM_000361, Version: NM_000361.2 GI:40288292; or Swiss-Prot: P07204.

[0006] When used herein, "derivative" refers to a variation or analog, or modification thereof, relative to a reference material. For example, a thrombomodulin derivative can refer to a mutant protein, truncation of a native protein sequence, or synthetically modified variant including modification by pegylation or conjugation such as to a polymer or surface. A derivative may employ the use of natural amino acids, non-natural amino acids, and/or other chemical moieties; whether covalently or non-covalently associated; and whether associated during translation, post-translation, or apart from translation such as in a synthetic approach; as disclosed herein or as would be understood in the art. In a particular embodiment, a derivative is a truncated, mutated, pegylated, conjugated thrombomodulin.

[0007] The following abbreviations are applicable. TM, thrombomodulin; PEG, polyethylene glycol.

[0008] It is recognized that regardless of the ultimate correctness of any mechanistic explanation or hypothesis, an embodiment of the invention can nonetheless be operative and useful.

[0009] In an embodiment, the present invention relates to the generation of novel thrombomodulin ("TM") conjugates, including soluble conjugates.

[00010] In an embodiment, the invention provides a method of site-specific PEGylation for a protein molecule. In a preferred embodiment, the protein molecule is bioactive thrombomodulin or a derivative thereof.

[00011] In an embodiment, a TM analog sequence comprises a catalytically active site capable of activating protein C (EGF 4-6 domains) and single or multiple non-natural amino acids. In a particular embodiment, TM analogs are conjugated to linear or branched natural or synthetic polymers via the non-natural amino acids. In an embodiment, the invention provides methods for conjugation of the TM conjugates to the surfaces of synthetic or natural materials, to targeting groups for site specific delivery of the agent, and/or to compounds that contain one or more additional anti-inflammatory/anti-thrombotic properties.

[00012] In an embodiment, a TM conjugate can be useful as a systemic agent for treatment of one or more conditions such as micro or macrovascular blood clots, stroke, heart attack, disseminated intravascular coagulation or other inflammatory or prothrombotic condition.

[00013] In an embodiment, the invention provides a coating of a surface of a medically implanted or human tissue or fluid contacting device. For example, implants or devices can include but are not restricted to: vascular grafts, stents, heart valves, dialysis membranes, membrane oxygenators, catheters, and guide wires to alter surface properties. A further embodiment of the invention provides a coating for living cells or tissues, including, but not restricted to smooth muscle cells, fibroblasts, endothelial cells, stem cells, chondrocytes, osteoblasts, pancreatic islets, or genetically engineered cells to establish or enhance the anti-inflammatory properties of the cells.

[00014] In an embodiment, the invention provides a covalent conjugation of truncated TM derivatives onto a blood or tissue contacting surface using natural or synthetic polymers as a spacer.

[00015] In an embodiment, the invention provides a novel method for covalent conjugation of TM to synthetic or natural materials site-specifically without loss of protein bioactivity or with substantial retention thereof. In a particular embodiment, the invention provides a relatively short recombinant TM construct containing EGF-like domains 4-6 and single or multiple non-natural amino acids optionally or preferably at the C-terminal portion of the construct. In a particular embodiment, the invention provides a modified TM through reaction with a suitable polymer spacer via one or more non-natural amino acids. In a particular embodiment, a TM is modified for further immobilization onto surfaces or for conjugation to linear or multifunctional natural or synthetic compounds that contain other anti-inflammatory or anti-thrombotic properties. In an embodiment, a bioconjugation reaction occurs in mild conditions to preserve TM bioactivity. In a preferred embodiment, polyethylene glycol ("PEG") is used as the polymer spacer.

[00016] In an embodiment, a modified construct is adapted for further immobilization onto a surface or for conjugation to linear or multifunctional natural or synthetic compounds that are capable of an anti-inflammatory or anti-thrombotic activity.

[00017] In an embodiment, PEGylation of a protein can confer one or more advantages, e.g. an increase in plasma half-life, stability against proteolytic cleavage, and a decrease of protein immunogenicity.

[00018] In an embodiment, the invention provides compositions including nucleic acid and protein molecules. In a particular embodiment, the compositions relate to sequences of the Table S1 below.

[00019] Table S1 of selected sequence listing information.

SEQ ID NO:	Brief Description	Type
1	TMb	DNA/RNA
2	automatic translation	PRT
3	TMb	PRT
4	human TM	PRT
5	PCR primer	DNA
6	PCR primer	DNA

[00020] In an embodiment, the invention provides methods and compositions relating to generation of thrombomodulin constructs comprising a non-natural amino acid. In an embodiment, a construct is thrombomodulin or a thrombomodulin derivative. In an embodiment, the invention provides recombinant expression or synthetic production of such constructs. In a preferred embodiment, the construct is generated by recombinant expression. In a preferred embodiment, the invention provides methods and compositions comprising an extracellular portion of thrombomodulin. In a preferred embodiment, the extracellular portion of thrombomodulin further comprises catalytically active sites. In a preferred embodiment, the extracellular portion of thrombomodulin is capable of activating protein C.

[00021] In a preferred embodiment, a thrombomodulin derivative comprises a single non-natural amino acid or multiple non-natural amino acids. In an embodiment, a non-natural amino acid can include those as would be understood in the art. For example, non-natural amino acids can include: methionine analogues, alanine analogues, phenylalanine analogues, leucine analogues, proline analogues and isoleucine analogues. An example of methionine analogues includes: L-2-amino-4-azido-butanoic acid.

[00022] In a preferred embodiment, a thrombomodulin derivative comprises a single non-natural amino acid at the C-terminal portion of the construct.

[00023] In an embodiment, the invention provides a thrombomodulin construct wherein the construct is conjugated to a natural or synthetic polymer or other natural molecule such as an antibody or other ligand recognition molecule. In an embodiment, the conjugation is via at least one non-natural amino acid in the recombinant protein.

[00024] In an embodiment, a synthetic polymer for conjugation to a construct can include a linear or branched synthetic polymer. For example, a linear or branched synthetic polymer can include: poly(t-butyl acrylate), poly(t-butyl methacrylate), polyacrylamide, glycolipid and their mimetics; and other polymers as would be understood in the art. Examples of a natural polymer include: glycoproteins and their mimetics, poly(arginine), polysaccharides and their mimetics; and other

polymers as would be understood in the art. In an embodiment, a ligand recognition molecule is antifibrin antibody.

[00025] In an embodiment, a construct is conjugated to a linear or branched poly(ethylene glycol) molecule. In a preferred embodiment, the construct is conjugated to linear poly(ethylene glycol).

[00026] In an embodiment, the invention provides a thrombomodulin construct conjugated to a natural or synthetic polymer for surface anchoring. In an embodiment, the natural or synthetic polymer is multifunctional. In a preferred embodiment, the construct is conjugated to poly(ethylene glycol) for surface anchoring of the conjugate. In an example, an anchoring group includes: biotin, conjugated diene, azide, alkyne, diphenylphosphine, triarylphosphine; and other groups as would be understood in the art. In an example, a surface targeting group includes: sialyl-Lewis X; an antibody, Fab fragment or the like, or other analogous protein or non-protein recognition molecule (including an aptamer) capable of recognizing VCAM-1, ICAM-1, or other inflammatory cell surface proteins; antifibrin antibody; streptavidin, azide, alkyne, N-(ε-maleimidocaproyl; and others as would be understood in the art.

[00027] In an embodiment, a construct is conjugated to a synthetic polymer for anchoring to a surface of a synthetic material or a natural material. In an example, synthetic materials include: poly(tetrafluoroethylene) , polysiloxanes, poly(ether urethane urea), poly(lactic acid-co-glycolic acid), a glass surface and derivatives; and other materials as would be understood in the art. In an example, natural materials include: cells, tissues, and blood vessels.

[00028] In an embodiment, the invention provides compositions and methods of a surface coating for a medically implanted or human tissue or fluid contacting device including but not restricted to vascular grafts, stents, heart valves, dialysis membranes, membrane oxygenators, catheters, or guide wires. In an embodiment, the surface coating alters a surface property of the implant or the device.

[00029] In an embodiment, the invention provides compositions and methods for coating a surface of living cells or tissues, including, but not restricted to smooth

muscle cells, fibroblasts, endothelial cells, stem cells, chondrocytes, osteoblasts, pancreatic islets, or genetically engineered cells.

[00030] In an embodiment, the invention provides a recombinant thrombomodulin construct conjugated to a multifunctional natural or synthetic polymer, wherein the polymer is capable of an anti-inflammatory or anti-thrombotic property. In an embodiment, the construct is conjugated to a synthetic polymer comprising one or more anti-inflammatory groups. In an embodiment, the synthetic polymer comprises one or more additional anti-inflammatory groups. In an example, an anti-inflammatory group includes sialic acids and their mimetics/derivatives; and other groups as would be understood in the art.

[00031] In an embodiment, the invention provides a construct conjugated to a synthetic polymer further comprising an anti-coagulant or anti-thrombotic group. In an example, an anti-coagulant or anti-thrombotic group includes heparin and its mimetics/derivatives; and other groups as would be understood in the art.

[00032] In an embodiment, the invention provides a systemic agent for treatment of a medical condition, wherein the condition relates to a microvascular or macrovascular blood clot, stroke, heart attack, disseminated intravascular coagulation, or other inflammatory or prothrombotic condition. In an embodiment, the invention provides a method of treatment of the medical condition by administering to a patient in need a construct of the invention.

[00033] In an embodiment, the invention provides a truncated thrombomodulin protein derivative comprising EGF (4-6) like domains, a substitution of Leucine for methionine at position 388, and a GGM amino acid motif appended at a carboxy terminus of said derivative. In an embodiment, the GGM protein motif is expressed as a protein motif with a non-natural amino acid corresponding to the M amino acid residue. In an embodiment, the invention provides SEQ ID NO:3.

[00034] In an embodiment, the invention provides a truncated thrombomodulin derivative conjugate comprising a truncated thrombomodulin derivative and a polymer; wherein the thrombomodulin derivative comprises EGF (4-6) like domains, a substitution of Leucine for methionine at position 388, and a GGM amino acid motif

appended at a carboxy terminus of said derivative. In an embodiment, the polymer comprises polyethylene glycol.

[00035] In an embodiment, the invention provides a truncated thrombomodulin nucleic acid derivative comprising EGF (4-6) like domains, a substitution of Leucine for methionine at position 388, and a nucleic acid sequence capable of encoding a Gly Gly Met motif appended at a carboxy terminus of said derivative. In an embodiment, the nucleic acid sequence comprises SEQ ID NO:1.

[00036] In an embodiment, the invention provides a method of generating a purified truncated thrombomodulin derivative protein, wherein the protein comprises EGF (4-6) like domains, a substitution of Leucine for methionine at position 388, and a non-natural amino acid; comprising the steps of providing a truncated thrombomodulin nucleic acid sequence; recombinantly expressing said nucleic acid sequence in the presence of a non-natural amino acid precursor; and purifying a recombinant expression product; thereby generating a purified truncated thrombomodulin derivative protein. In an embodiment, the nucleic acid sequence is SEQ ID NO:1.

BRIEF DESCRIPTION OF THE FIGURES

[00037] Scheme A. Synthesis of the methyl-PEG-triarylphosphine conjugate 2 and its conjugation to azido-functionalized TMB.

[00038] Figure 1 is a schematic structure of the targeted truncated TM, whose amino-acid sequence is encoded by the gene TM_{GGM}.

[00039] Figure 2 illustrates results of Western blot analysis of TM derivatives (4-20% SDS-PAGE gel): (1) purified $\mathbf{TM_A}$; (2) enterokinase cleavage of $\mathbf{TM_A}$ leading to the target protein $\mathbf{TM_B}$; (3-5) conjugation of **2** to $\mathbf{TM_B}$ over time: 4h, 8h and 24h, respectively. M: Molecular weight marker proteins.

[00040] Figure 3 illustrates MALDI-TOF Mass Spectra of (a) the starting polymer methyl-PEG-amine (average molecular weight detected: 5,668 D) and (b) the

methyl-PEG-triarylphosphine conjugate **2** (average molecular weight detected: 6,034 D, calculated 6014 D).

[00041] Figure 4 illustrates (a) Western blot analysis; and (b) barium chloride/iodine staining of a 10% SDS-PAGE gel: (1) and (3) reaction mixture of the bioconjugation of **2** to $\mathbf{TM_B}$ after 36h; (2) initial azido-functionalized $\mathbf{TM_B}$. M: Molecular weight marker proteins.

[00042] Figure 5 illustrates a genetically directed synthesis using non-natural amino acids and a conjugation reaction.

[00043] Figure 6 illustrates bioorthogonal surface ligation of azidolactose (A) and azidoTM (B) via azide-alkyne [3+2] cycloaddition ("Click" chemistry). Surface bound carbohydrates and TM were visualized by staining with a FITC-lectin (A) and FITC anti-S-tag MAb (B), respectively.

DETAILED DESCRIPTION OF THE INVENTION

[00044] The invention may be further understood by the following non-limiting examples.

[00045] EXAMPLE 1. C-Terminal site-specific PEGylation of a truncated thrombomodulin with full bioactivity

[00046] Abstract: Addition of polyethylene glycol to bioactive proteins (PEGylation) can improve their plasma half-life, enhance stability against proteolytic cleavage, and may also decrease protein immunogenicity. PEGylation can involve reaction to available lysine amino groups, some of which may be within or near a bioactive site. Often PEGylation protocols are nonspecific and result in a loss of protein activity. We disclose a strategy for site-specific PEGylation of a thrombomodulin derivative (TM) at the C-terminus. A truncated TM mutant consisting of epidermal growth factor (EGF)-like domains 4-6 was expressed using recombinant techniques in *Escherichia coli* with a C-terminal azido-methionine. The TM mutant was site-specifically conjugated to a methyl-PEG-triarylphosphine compound via Staudinger reaction. Enzymatic activity of the TM construct before and after PEGylation was

substantially similar, which confirms the utility of this site-specific PEGylation method and molecules thereby produced.

[00047] Introduction: Pegylation of proteins increases both their molecular size and steric hindrance, which can result in an increase of protein plasma half-life and resistance to proteolytic cleavage. In addition, protein immunogenicity may be decreased (1,2). Characteristically, PEGylation usually involves reaction to available lysine amino groups, some of which may be within or near a bioactive site. Thus, protocols are often nonspecific and result in a loss of protein activity (3,4). For example, Han *et al.* (5) conjugated TM to PEG via trichlorotriazine as a coupling agent for immobilization onto a glass surface. Despite successful immobilization, reduced TM activity was noted presumably due to alteration of protein conformation after PEGylation. To overcome the limitations of current conjugation strategies, several approaches have been proposed. Site-specific PEGylation can be achieved through the introduction of a cysteine residue in engineered proteins with a free thiol available for conjugation reactions (6,7). As a consequence, several PEG derivatives have been developed for that purpose (8,9,10,11). The efficacy of this approach is compromised, however, by a low yield of PEGylated protein and often by a substantial loss of activity, for example if abnormal protein folding is induced by the introduction of the cysteine residue (12). As an alternative strategy, Yamamoto *et al.* (13) reported site-specific PEGylation of a lysine-deficient tumor necrosis factor-α at its N-terminus.

[00048] We report herein the novel use of a strategy for site-specific PEGylation of human thrombomodulin. This transmembrane protein is a critical regulator of the protein C pathway and represents a major anticoagulant mechanism that is operative under physiologic conditions *in vivo* (14,15). TM is a cofactor for thrombin-catalyzed activation of protein C, enhancing the rate of the reaction by 1000-fold (16). In order to closely mimic the TM structure as it appears at the cell surface (17,18), and consequently preserve its bioactivity, we investigated the PEGylation at the C terminus. Using a genetically-directed synthesis in *Escherichia coli*, we first expressed a short TM construct containing EGF-like domains 4-6 and an azido-functionalized methionine analog (19) as a C-terminal linker. The PEGylation was

then achieved through Staudinger ligation (20) with a suitably engineered PEG derivative.

[00049] EXPERIMENTAL PROCEDURES

[00050] Materials. All chemical reagents were obtained from Sigma Chemical Corporation (St. Louis, MS). The methyl-PEG-amine 5,000 was purchased from Netkar Corp. (Hunstville, AL). The BamHI and Shrimp Alkaline Phosphatase enzymes were obtained from New England Biolabs, Inc. (Beverly, MA). The Quikchange Site-Directed Mutagenesis kit was from Stratagene (La Jolla, CA). E.coli strain B834 (DE3), plasmid pET-39b(+), S-Tag Rapid Assay kit and Site-Specific Enterokinase Cleavage and Capture Kits were from Novagen (Madison, WI). All plasmid purification kits were purchased from QIAGEN Inc. (Chatsworth, CA). The mouse monoclonal antibody to human thrombomodulin was from COVANCE Corp. (Richmond, CA). Synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Purified recombinant human PC and human α thrombin were from Haematologic Technologies Inc. (Essex Junction, VT). Human anti-thrombin, recombinant human Thrombomodulin (soluble truncated form of thrombomodulin that lacks the putative transmembrane and the cytoplasmic domains, approximate weight 68 kD) and chromogenic substrate SPECTROZYME PCa were purchased from American Diagnostica Inc. (Stamford, CT). All reagents for manipulating DNA and bacteria were sterilized by autoclave.

[00051] Instrumentation. MALDI-TOF mass spectrometry data were performed on an Applied Biosystem Voyager-DE™ STR Biospectrometry™ Workstation MALDI-TOF Mass Spectrometer using an 2-(4-hydroxy-phenylazo)benzoic acid matrix. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 600 MHz (^{1}H, ^{13}C) and 242 MHz (^{31}P) on a Varian INOVA in $CDCl_3$ (internal Me_4Si, δ (delta) ppm). Optical density was recorded on a Varian Cary 50 Bio UV-visible spectrophotometer.

[00052] Synthetic Gene Construction. A DNA fragment encoding for EGF (4-6) domains of human TM was obtained by polymerase chain reaction using primers 5'-TACCCTAACTACGACCTGGTG-3' (SEQ ID NO:5) and 5'-TATGAGCAAGCCCGAATG-3' (SEQ ID NO:6). Through a series of intermediate constructs, this fragment was used to generate a gene containing a Leucine (Leu)

substitution for Methionine-388 (Met-388), N-terminal and C-terminal BamH I sites and a C-terminal linker GlyGlyMet using site-directed mutagenesis. The final construct (TM_{GGM}) was then inserted using the BamH I site of the expression plasmid pET-39b(+). All mutations were verified through sequence analysis.

[00053] Protein expression and purification. pET39b(+)-TM_{GGM} was transformed into the *E. coli* methionine auxotroph B834(DE3). M9 minimal medium (500 mL) supplemented with 1 mM $MgSO_4$, 0.4 wt% glucose, 1 mg/L thiamine chloride, 0.1 mM $CaCl_2$, kanamycin (30 mg/L) and all proteinogenic amino acids (40mg/L) was inoculated with 20 mL of an overnight culture of the transformed cells. When the turbidity of the culture reached an OD_{600} of 0.8, protein expression was induced by addition of isopropyl-β (beta)-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. After 5min, the medium was exchanged to remove methionine, cells were sedimented (4000g, 20min), and the cell pellet washed twice with 200 mL of 1xM9 salts. Cells were resuspended in 500 mL of the M9 minimal medium described above, without methionine but supplemented with 100 mg/L of azido-functionalized methionine analog (19). A culture lacking methionine served as the negative control. Cultures were grown for 4.5 h at 37 °C.

[00054] The expression of the TM protein was analyzed by 4-20% gradient SDS-PAGE gel electrophoresis and visualized by Western blot analysis using a mouse monoclonal antibody to human thrombomodulin. The target protein was expressed as a N-terminal Dsba enzyme fusion to a leader sequence containing an *Enterokinase* cleavage site, hexahistidine, and S-tags (protein **TM_A**). S-Tag Rapid Assay was used to quantify protein concentration and therefore expression yield, which averaged 17 mg/L of cell culture. **TM_A** was purified from the cell pellet by using immobilized metal-affinity chromatography on TALON resin (Clontech Laboratories, Inc.) under native conditions using an imidazole gradient for elution of the target polypeptide. The cells were first harvested by centrifugation at 4 °C at 10,000g for 30 min and resuspended in 25 mL of lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10% glycerol, 1 mg/mL lysozyme, 10 μg/mL PMSF, pH 8). After incubation on ice for 30min, the cell lysate was clarified by centrifugation at 10,000g for 20 min. The soluble extract was then loaded onto a column containing TALON metal affinity resin (25 mL), which had been preequilibrated with lysis buffer. The

weakly binding proteins were removed by rinsing the column with 125 mL wash buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10% glycerol, 20mM imidazole, pH 8). **TM_A** was eluted by the addition of 50 mL of elution buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10% glycerol, 250mM imidazole, pH 8). The chromatographic fractions were analyzed by 4-20% gradient SDS-PAGE gel electrophoresis and visualized by Western blot analysis using mouse monoclonal antibody to human thrombomodulin. The nitrocellulose membrane was developed using the ECL plus Western blotting detection kit (Amersham Biosciences, UK). *Enterokinase* cleavage removed the fusion tag and generated the target protein (**TM_B**). N-terminal sequencing, amino acid compositional and mass analysis (SELDI-TOF) confirmed the integrity of **TM_B**: (Mass detected (m/z): 16,545.2 D (calculated 16,540.1 D)).

[00055] Synthesis of methyl-PEG-triarylphosphine (2). 1,3-Dicyclohexylcarbodiimide (DCC) (5.7 mg, 27 μmol) was added to a solution of phosphino reagent (**1**) (10.3 mg, 28 μmol) in anhydrous $CHCl_3$ (2 mL) at room temperature under argon. The mixture was stirred for 1h, followed by the addition of methyl-PEG-amine (m-PEG-NH_2 (M_n 5kD)) (120 mg, 24 μmol) dissolved in $CHCl_3$ (2 mL). The mixture was subsequently agitated for 2.5 days under argon. The methyl-PEG-triarylphosphine conjugate was recovered by precipitation in diethyl ether (200 mL) and filtration. The precipitate was dissolved in water. The aqueous solution was filtrated and lyophilized to afford the final product with 50% of substitution. The product was used in the Staudinger reaction without further purification. ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.38 (s, 3H, O-CH_3 (PEG)), 3.45-3.90 (m, 448 H, O-CH_2 (PEG)), 7.47–7.55 (m, 6H), 7.67-7.70 (m, 5H), 7.91-7.94 (m, 1H), 8.12-8.13 (m, 1H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 40.0, 41.3, 52.5, 59.3, 70.2, 70.6, 71.1, 72.0, 128.5, 128.7, 128.8, 130.5, 131.0, 131.3, 132.0, 133.3, 134.1, 137.7, 138.1, 166.5, 166.7. ^{31}P NMR (242 MHz, $CDCl_3$): δ-3.11. MALDI-TOF: 6034 D (calculated 6014 D).

[00056] **TM-PEG Conjugation**. To a solution of **TM_B** in Phosphate Buffered Saline pH 7.4 (300-500 nM, 100 μL) was added a large excess (1 mg) of lyophilized methyl-PEG-triarylphosphine and the mixture was subsequently heated at 37°C for 36 h to obtain maximum conjugation. The mixture was divided in half and two sets of reactions were subjected to SDS/PAGE in parallel. Proteins from the first gel were transferred to nitrocellulose membrane and visualized by Western blot analysis using

a mouse monoclonal antibody to human thrombomodulin. This procedure monitored the presence of TM_B proteins that had reacted with the phosphine, and were therefore PEG-labeled, as well as unreacted TM_B. The second gel was stained with barium chloride/iodine solutions to reveal PEGylated molecules only (21).

[00057] **TM enzymatic activity assay**. A saturated concentration of **TM_B** in 20 µL of assay buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 2.5 mM $CaCl_2$) was incubated for 30 min at 37 °C with 10 nM human α thrombin and varying amounts of human protein C (0-8 µM). Reactions were quenched by addition of 5 µL of human antithrombin (10.7 µM) and incubation for 5 min at room temperature. The amount of activated protein C formed was determined by addition of 275 µL of SPECTROZYME PCa (218 µM), followed by incubation for 20 min at room temperature. SPECTROZYME PCa is a chromogenic substrate used for the quantification of activated protein C in solution. One mole of the substrate is hydrolyzed by activated protein C producing one mole of p-nitroaniline (pNA). The pNA concentration was determined by UV spectrophometry at 407nm using the equation:

[00058] $OD_{407nm} = -0.0014 + 0.0096\ [pNA]$ (1)

[00059] where equation (1) was obtained by optical density measurements of pNA solutions of known concentrations. The corresponding rate (mol/min) of p-nitroaniline formed (R_{pNA}) was obtained by dividing the pNA concentration by the 20 min assay time.

[00060] A second standard plot was used to determine the activated protein C concentration from R_{pNA}, where

[00061] $R_{pNA} = -0.059 + 0.0671\ [APC]$ (2)

[00062] To obtain equation (2), commercial activated protein C was dissolved in assay buffer at various concentrations and incubated with Spectrozyme PCa for 20 min at room temperature. The corresponding rate (mol/min) of activated protein C formed (R_{APC}) by the TM-thrombin complex was obtained by dividing the observed APC concentration by the 30 min assay time.

[00063] Values obtained for control experiments without TM were subtracted to give final rates of activated protein C produced. Michaelis-Menten parameters (k_{cat}, K_m) were calculated from a plot of R_{APC} versus protein C concentration.

[00064] RESULTS

[00065] Our approach utilized the three consecutive EGF-like domains 4-6 (EGF4-6) of human TM. We analyzed that the corresponding amino-acid sequence contains only one methionine residue (Met-388) and considered its possible impact on protein bioactivity. We also noted that the mutation of Met-388 to leucine (Leu) could contribute resistance to oxidative inactivation while allowing the potential for greater enzymatic activity than the native TM protein. We therefore have made the significant finding that a truncated TM fragment containing EGF domains 4-6 with the insertion of a C-terminal non-natural methionine analog can provide a good target for site-specific PEGylation.

[00066] To create a short recombinant bioactive TM mutant, we first used the DNA fragment encoding for the amino-acids sequence 349 to 492 to generate a gene containing a Met-388-Leu substitution and a C-terminal linker GlyGlyMet using site-directed mutagenesis (**Figure 1**). The final construct (TM_{GGM}) was then inserted in the expression plasmid pET-39b(+). This plasmid contains a leader gene sequence coding for the *Dsba* enzyme, which is a periplasmic enzyme catalyzing the formation and isomerization of disulfide bonds of expressed proteins. Therefore, we expected the target TM mutant to possess all disulfide bonds required for proper protein folding and enzyme activity. Expression under the induction of IPTG using the *E. coli* methionine auxotroph B834 (DE3)/pET39b(+)-$\boldsymbol{TM_{GGM}}$ in cultures depleted of methionine and supplemented with an azido-functionalized methionine analog, azidohomoalanine (19), afforded the target protein as a N-terminal Dsba enzyme fusion to a leader sequence containing *enterokinase* recognition sequence, hexahistidine and S-tags. Protein expression was monitored by SDS/PAGE analysis and visualized by Western blot analysis. The target protein was not observed in negative control culture, whereas TM fused to the leader sequence ($\mathbf{TM_A}$) was clearly detected in positive control cultures supplemented with azidohomoalanine. The accumulation of $\mathbf{TM_A}$ was taken as preliminary evidence for incorporation of the non-natural amino acid. $\mathbf{TM_A}$ was purified from the cell pellet by using immobilized metal-

affinity chromatography with stepwise imidazole gradient elution under native conditions. *Enterokinase* cleavage removed the fusion tag and generated the target proteins (**TM_B**). N-terminal sequencing, amino acid compositional and mass analysis (mass detected: 16,545D) confirmed the integrity of **TM_B**. **TM_B** was characterized by a band at 33kD on Western blot analysis (Figure 2), which is consistent with TM associating into dimers even under denaturing conditions (25).

[00067] Having expressed an azide-modified form of TM, we then investigated the selective Staudinger ligation of this protein with a methyl-PEG-triarylphosphine conjugate **2** (**Scheme A**). We choose this ligation protocol in order to minimize the risk of side reactions that might alter TM structure and activity. In its classical form, the Staudinger reaction meets many of the criteria required of a chemoselective ligation in a cellular environment. The phosphine and the azide react rapidly, selectively, and in high yield, in water at room temperature. We used **1** in the synthesis of methyl-PEG-triarylphosphine conjugate **2** (see Kiick *et al.* (19); preparation of a triarylphosphine compound **1** that was used for the preparation of a protein-Flag conjugate, as well as for labeling cell surface azide-bearing sialic acids with a biotinylated phosphine (20)).

[00068] The carboxylic acid group of **1** was reacted with the amino group of a commercially available derivative, methyl-PEG-amine (m-PEG-NH_2 (5kD)) with amide bond formation (**Scheme A**). The incorporation of a phosphino group in PEG was assessed by ^{1}H, ^{13}C, and ^{31}P NMR, as well as by MALDI-TOF spectroscopy (**Figure 3**). The average molecular weight of 6,034 D observed for the methyl-PEG-triarylphosphine conjugate **2** is consistent with an expected mass increase of 346 D, as a result of linking the phosphino compound **1** to m-PEG-NH_2 (average molecular weight observed: 5,668 D).

[00069] The addition of m-PEG-NH_2 to **1** dramatically enhances the water solubility of the hydrophobic phosphino group. A large excess of lyophilized methyl-PEG-triarylphosphine conjugate **2** (1 mg) was added to 100 μL of an aqueous solution of **TM_B** in PBS at pH 7.4 and Staudinger ligation performed at 37 °C for 24h. The formation of the **TM_B**-PEG conjugate was followed by Western blot analysis of the reaction mixture over time (**Figure 2**). The gel shows a band at higher molecular weight than the initial **TM_B** with a simultaneous disappearance of the **TM_B** band.

These results indicate that the Staudinger reaction proceeded efficiently with incorporation of the PEG polymer into the TM construct. As the reaction was not complete after 24h, we allowed it to proceed for an additional 12h. Western blot analysis (**Figure 4a**) and barium chloride/iodine staining (**Figure 4b**) of SDS-PAGE gels were performed in parallel to monitor the formation of the **TM_B**-PEG conjugate. Disappearance of the band characteristic of the unconjugated **TM_B** revealed that bioconjugation of **TM_B** to PEG was substantially (approximately 100%) complete. The SDS gel run in parallel under identical conditions but stained with barium chloride/iodine confirmed the presence of PEG in the bioconjugate. The presence of a low molecular weight band represents unreacted methyl-PEG-triarylphosphine **2**.

[00070] Enzyme activity was assessed to determine the effect of PEGylation on TM cofactor activity (**Table 1**).

[00071] Table 1. Michaelis-Menten parameters for protein C activation by TM derivatives.

Parameter	TM_A	TM_B	PEG-TM_B	Commercial TM
K_M (μM)	0.9 ± 0.2	1.0 ± 0.5	1.0 ± 0.5	0.7 ± 0.1
k_{cat} (min^{-1})	0.22 ± 0.05	0.16 ± 0.05	0.20 ± 0.05	0.14 ± 0.02
k_{cat}/K_M (min^{-1}. μM^{-1})	0.26 ± 0.10	0.16 ± 0.05	0.20 ± 0.05	0.21 ± 0.05

[00072] We initially investigated the activity of **TM_A** and **TM_B** mutants, as well as a commercial recombinant human TM mutant consisting solely of the extracellular domain (American Diagnostica Inc.). Clarke et al. (23) have reported that the Met-388-Leu mutation results in a 2-fold increase in k_{cat} for the activation of protein C by a thrombin-TM fragment complex. Although we were unable to observe this enhancement of activity, Michaelis-Menten parameters were similar for **TM_A** and **TM_B**, as well as for the commercial human TM protein. We inferred that the generated TM mutants exhibited the requisite conformation and structure necessary for proper thrombin binding and protein C activation. Significantly, the incorporation of PEG into TM_B did not affect cofactor activity, indicating that this site-specific PEGylation scheme did not interfere with thrombin binding and protein C activation. Parenthetically, despite a concerted effort by our group, previous attempts to

generate an enzymatically active truncated TM mutant with a C terminal cysteine, as a potential bioconjugation site, were unsuccessful using both yeast and *E. coli* expression systems. We speculate that an additional terminal cysteine likely interfered with normal protein folding dependent upon the presence of native disulfide loops in the EGF sequences.

[00073] CONCLUSIONS

[00074] A human thrombomodulin derivative containing EGF(4-6)-like domains designed with an azido-functionalized methionine C-terminal linker was successfully synthesized using a genetic engineering strategy. The protein exhibited bioactivity towards protein C that was comparable to native human TM. The TM construct was successfully conjugated to a novel engineered methyl-PEG-triarylphosphine compound *via* Staudinger reaction under mild conditions. Enzyme activity before and after PEGylation was substantially similar indicating the utility and successful application of the site-specific PEGylation method. To our knowledge, this report is the first to describe site-specific PEGylation of a thrombomodulin mutant with retention of substantially full bioactivity.

[00075] References for Example 1:

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[00076] EXAMPLE 2. Synthesis of a recombinant thrombomodulin conjugate for immobilization onto a thin film.

[00077] Covalent immobilization of TM onto polymeric surfaces has been investigated (AD, AE). In all cases, the conjugation scheme utilized a non-site specific carbodiimide based coupling reaction in which TM was coupled to the substrate via any freely available amino or carboxyl functionality on the protein surface. Consequently, TM bioactivity was often reduced after surface coupling.

[00078] We have demonstrated that full length TM can be incorporated into a stable, membrane-mimetic thin film over a wide range of surface concentrations by a process of lipid/protein self-assembly and *in situ* photopolymerization. See AA, AB, AC. However, as an alternate strategy using genetically directed synthesis, we have created a short TM construct containing the catalytic region of EGF domains 4-6 along with an artificial amino acid analog, azido (N3)-alanine, incorporated biosynthetically at the C-terminus of the protein (rTM-N3). Through Staudinger ligation with a suitable phosphine PEO derivative (MW 3000), we have generated TM-PEO conjugates. The azidoTM construct and conjugate have been fully characterized by Western blotting and SDSPAGE. The catalytic activity (kcat, Km) of the conjugate is comparable to the rTM-N3 mutant alone, as well as a commercially available soluble human TM protein (Solulin™). Of note, by using a biotin or diene terminated PEG derivative, for example, TM can then be rapidly and directly coupled to a surface, which provides an additional approach for linking TM to membrane assemblies.

[00079] Staudinger ligation of azidoTM with phosphine-PEO is not without limitations. The requisite phosphines are susceptible to air oxidation, and their

optimization for improved water solubility and increased reaction rate is synthetically challenging. Thus, we have investigated alternate strategies for direct single-step bioorthogonal coupling of rTM-N3 to target surfaces. Specifically, we have demonstrated that rTM-N3 can be coupled to surfaces via an azide-alkyne [3+2] cycloaddition, termed "click" chemistry (AF, AG). Significantly, both this approach and Staudinger ligation provide a means to selectively conjugate complex biomolecules in richly functionalized environs under mild conditions that do not alter biomolecular activity. The chemical conjugation scheme is summarized in Figure 6. See (AF).

[00080] The rTM-N3 was expressed with an N-terminal S-tag, which allowed the effectiveness of surface coupling to be evaluated using a FITC-labeled anti-S-tag antibody. Likewise, the feasibility of using this strategy to link N3-derivatized carbohydrates to target surfaces was also evaluated using azidolactose, as a model oligosaccharide for more complex polysaccharides, such as heparin. A FITC-labeled lectin was used to ascertain the extent and homogeneity of carbohydrate surface conjugation. This scheme proved quite versatile for surface coupling of both rTM and carbohydrates.

[00081] References for Example 2:

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[00082] STATEMENTS REGARDING INCORPORATION BY REFERENCE AND VARIATIONS

[00083] All references throughout this application, for example patent documents including issued or granted patents or equivalents; patent application publications; and non-patent literature documents or other source material; are hereby incorporated by reference herein in their entireties, as though individually incorporated by reference, to the extent each reference is at least partially not inconsistent with the disclosure in this application (for example, a reference that is partially inconsistent is incorporated by reference except for the partially inconsistent portion of the reference).

[00084] Any appendix or appendices hereto are incorporated by reference as part of the specification and/or drawings.

[00085] Compositions including compounds of the invention can have prodrug forms. Prodrugs of the compounds of the invention are useful in the methods of this invention. Any compound that will be converted in vivo to provide a biologically, pharmaceutically or therapeutically active form of a compound of the invention is a prodrug. Various examples and forms of prodrugs are well known in the art. A biomolecule such as a precursor protein or precursor nucleic acid can be a prodrug. Examples of prodrugs are found, inter alia, in Design of Prodrugs, edited by H. Bundgaard, (Elsevier, 1985), Methods in Enzymology, Vol. 42, at pp. 309-396, edited

by K. Widder, et. al. (Academic Press, 1985); A Textbook of Drug Design and Development, edited by Krosgaard-Larsen and H. Bundgaard, Chapter 5, "Design and Application of Prodrugs," by H. Bundgaard, at pp. 113-191, 1991); H. Bundgaard, Advanced Drug Delivery Reviews, Vol. 8, p. 1-38 (1992); H. Bundgaard, et al., Journal of Pharmaceutical Sciences, Vol. 77, p. 285 (1988); and Nogrady (1985) Medicinal Chemistry A Biochemical Approach, Oxford University Press, New York, pages 388-392).

[00086] Where the terms "comprise", "comprises", "comprised", or "comprising" are used herein, they are to be interpreted as specifying the presence of the stated features, integers, steps, or components referred to, but not to preclude the presence or addition of one or more other feature, integer, step, component, or group thereof. Separate embodiments of the invention are also intended to be encompassed wherein the terms "comprising" or "comprise(s)" or "comprised" are optionally replaced with the terms, analogous in grammar, e.g.; "consisting/consist(s)" or "consisting essentially of/consist(s) essentially of" to thereby describe further embodiments that are not necessarily coextensive.

[00087] The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention. It will be apparent to one of ordinary skill in the art that compositions, methods, devices, device elements, materials, procedures and techniques other than those specifically described herein can be applied to the practice of the invention as broadly disclosed herein without resort to undue experimentation. All art-known functional equivalents of compositions, methods, devices, device elements, materials, procedures and techniques described herein are intended to be encompassed by this invention. Whenever a range is disclosed, all subranges and individual values are intended to be encompassed. This invention is not to be limited by the embodiments disclosed, including any shown in the drawings or exemplified in the specification, which are given by way of example or illustration and not of limitation. The scope of the invention shall be limited only by the claims.

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1. A truncated thrombomodulin protein derivative comprising EGF (4-6) like domains, a substitution of Leucine for methionine at position 388, and a GGM amino acid motif appended at a carboxy terminus of said derivative.

2. The truncated thrombomodulin protein of claim 1 wherein said GGM protein motif is expressed as a protein motif with a non-natural amino acid corresponding to the M amino acid residue.

3. SEQ ID NO:3.

4. A truncated thrombomodulin derivative conjugate comprising a truncated thrombomodulin derivative and a polymer; wherein the thrombomodulin derivative comprises EGF (4-6) like domains, a substitution of Leucine for methionine at position 388, and a GGM amino acid motif appended at a carboxy terminus of said derivative.

5. The conjugate of claim 4 wherein the polymer comprises polyethylene glycol.

6. A truncated thrombomodulin nucleic acid derivative comprising EGF (4-6) like domains, a substitution of Leucine for methionine at position 388, and a nucleic acid sequence capable of encoding a Gly Gly Met motif appended at a carboxy terminus of said derivative.

7. The thrombomodulin derivative of claim 5 comprising SEQ ID NO:1.

8. A method of generating a purified truncated thrombomodulin derivative protein, wherein the protein comprises EGF (4-6) like domains, a substitution of Leucine for methionine at position 388, and a non-natural amino acid; comprising the steps of providing a truncated thrombomodulin nucleic acid sequence; recombinantly expressing said nucleic acid sequence in the presence of a non-natural amino acid precursor; and purifying a recombinant expression product; thereby generating a purified truncated thrombomodulin derivative protein.

9. The method of claim 8 wherein said nucleic acid sequence is SEQ ID NO:1.

10. The method of claim 8 wherein the non-natural amino acid is selected from the group consisting of: methionine analogues, alanine analogues, phenylalanine analogues, leucine analogues, proline analogues and isoleucine analogues.

11. The method of claim 10 wherein said methionine analog is L-2-amino-4-azido-butanoic acid.

12. The method of claim 8 wherein the non-natural amino acid is located at a C-terminal portion of the construct.

13. A method of site-specific PEGylation of a bioactive protein, comprising identifying an amino acid residue capable of alteration wherein the alteration does not substantially impair a protein activity; altering said amino acid residue; integrating a non-natural amino acid residue into said bioactive protein at a site, and conjugating a PEG polymer to said non-natural amino acid at the site.

14. The method of claim 13 wherein the bioactive protein is thrombomodulin.

15. The method of claim 13 wherein the bioactive protein is a thrombomodulin derivative.

16. A conjugate of a thrombomodulin protein or a thrombomodulin derivative and a polymer.

17. The conjugate of claim 16 wherein the polymer is PEG.

18. The conjugate of claim 16 wherein the polymer can confer a property for the conjugate selected from the group consisting of: an increase in plasma half-life, stability against proteolytic cleavage, and a decrease of protein immunogenicity, or combination thereof.

19. The conjugate of claim 16 wherein the conjugate is soluble.

20. A thrombomodulin derivative comprising a catalytically active site capable of activating protein C and a non-natural amino acid.

21. The thrombomodulin derivative of claim 20 wherein the derivative comprises an extracellular portion of thrombomodulin.

22. The thrombomodulin derivative of claim 20 wherein said active site comprises EGF (4-6) domains.

23. The thrombomodulin derivative of claim 20 conjugated via said non-natural amino acid to a linear or branched natural or synthetic polymer.

24. The derivative of claim 23 wherein said linear or branched synthetic polymer is selected from the group consisting of poly(t-butyl acrylate), poly(t-butyl methacrylate), polyacrylamide, glycolipid and their mimetics; and other polymers; glycoproteins and their mimetics, poly(arginine), polysaccharides and their mimetics; and other polymers as would be understood in the art.

25. A method of conjugating a thrombomodulin derivative to a substrate selected from the group consisting of: a surface of a synthetic or a natural material; a targeting group for tissue-specific or site-specific delivery of the conjugate; and a compound capable of one or more additional anti-inflammatory/anti-thrombotic properties.

26. A thrombomodulin derivative, or conjugate thereof, capable of acting as a systemic agent for treatment of one or more conditions selected from the group consisting of: micro or macrovascular blood clots, stroke, heart attack, disseminated intravascular coagulation, and other inflammatory or prothrombotic condition.

27. A coating of a surface of a medically implanted or human tissue or fluid contacting device comprising a thrombomodulin derivative or conjugate thereof.

28. The coating of claim 27 wherein the implanted or human tissue or fluid contacting device is selected from the group consisting of a vascular graft,

stent, heart valve, dialysis membrane, membrane oxygenator, catheter, and guide wire.

29. A coating of a coating for living cells or tissue, comprising a thrombomodulin derivative or conjugate thereof.

30. The coating of claim 29 wherein said cells or tissue is selected from the group consisting of smooth muscle cells, fibroblasts, endothelial cells, stem cells, chondrocytes, osteoblasts, pancreatic islets, genetically engineered cells, and other cells.

31. The coating of claim 27 wherein said coating is capable of establishing or enhancing an anti-inflammatory property of said cells or tissue.

32. A covalent conjugate of a truncated TM derivative and a blood or tissue contacting surface, wherein said conjugate comprises a natural or synthetic polymers as a spacer.

33. A method for covalent conjugation of thrombomodulin to a synthetic or natural material site-specifically with substantial retention of protein bioactivity.

34. A recombinant thrombomodulin TM construct comprising EGF-like domains 4-6 and single or multiple non-natural amino acids optionally at a C-terminal portion of the construct.

35. The construct of claim 34 wherein the construct is modified through reaction with a suitable polymer spacer via one or more non-natural amino acids.

36. The modified construct of claim 35 wherein the modified construct is adapted for further immobilization onto a surface or for conjugation to linear or multifunctional natural or synthetic compounds that are capable of an anti-inflammatory or anti-thrombotic activity.

37. A method of generating a bioconjugate of a TM or TM derivative comprising a mild conjugation reaction so as to preserve TM bioactivity.

38. A thrombomodulin construct wherein the construct is conjugated to a natural or synthetic polymer; or other natural molecule such as an antibody or other ligand recognition molecule.

39. The construct of claim 38 wherein the ligand recognition molecule is antifibrin antibody.

40. The conjugated construct of claim 38 wherein a conjugated potion is connected via at least one non-natural amino acid in a recombinant TM protein.

41. A construct conjugated to a poly(ethylene glycol) molecule, wherein the PEG molecule is linear or branched.

42. The construct of claim 41 wherein the PEG is linear.

43. A thrombomodulin construct conjugated to a natural or synthetic polymer for surface anchoring.

44. The construct of claim 43 wherein the natural or synthetic polymer is multifunctional.

45. The construct of claim 43 wherein the polymer is poly(ethylene glycol).

46. The construct of claim 43 further comprising an anchoring group selected from the group consisting of: biotin, conjugated diene, azide, alkyne, diphenylphosphine, triarylphosphine; and other groups as would be understood in the art.

47. The construct of claim 43 further comprising a targeting group selected from the group consisting of: sialyl-Lewis X; an antibody, Fab fragment or the like; a protein or non-protein recognition molecule (including an aptamer) capable of recognizing VCAM-1, ICAM-1, or other inflammatory cell surface proteins; antifibrin antibody; streptavidin; azide; alkyne; N-(ε-maleimidocaproyl; and other targeting group as would be understood in the art.

48. A TM construct conjugated to a synthetic polymer for anchoring to a surface of a synthetic material or a natural material.

49. The construct of claim 48 wherein a synthetic material is selected from the group consisting of: poly(tetrafluoroethylene) , polysiloxanes, poly(ether urethane urea), poly(lactic acid-co-glycolic acid), a glass surface and derivatives; and other materials as would be understood in the art.

50. The construct of claim 48 wherein a natural material is selected from the group consisting of cells, tissues, and blood vessels.

51. A surface coating for a medically implanted or human tissue or fluid contacting device comprising a TM construct or conjugate thereof.

52. A surface coating for living cells or tissues comprising a TM construct or conjugate thereof.

53. A recombinant thrombomodulin construct conjugated to a multifunctional natural or synthetic polymer, wherein the polymer is capable of an anti-inflammatory or anti-thrombotic property.

54. The construct of claim 53, wherein the synthetic polymer comprises one or more anti-inflammatory groups or one or more additional anti-inflammatory groups.

55. The construct of claim 54, wherein said anti-inflammatory group comprises sialic acids; mimetics/derivatives thereof; and other groups as would be understood in the art.

56. A TM construct conjugated to a synthetic polymer further comprising an anti-coagulant or anti-thrombotic group.

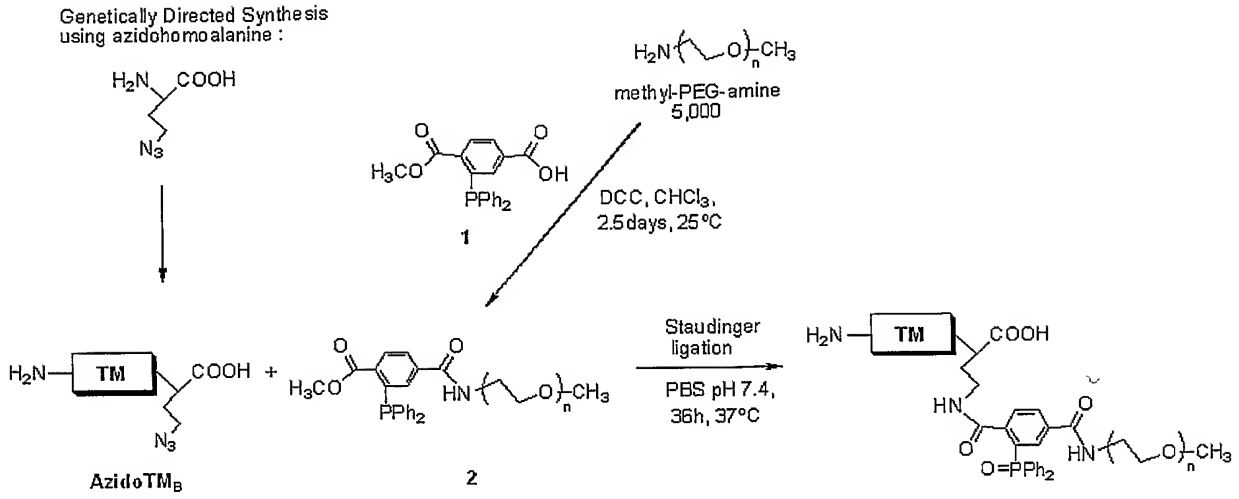
57. The construct of claim 56 wherein the group comprises heparin; mimetics/derivatives thereof; and other groups as would be understood in the art.

58. A systemic agent for treatment of a medical condition, wherein the agent comprises a TM construct or conjugate thereof; wherein the condition relates to a microvascular or macrovascular blood clot, stroke, heart attack,

disseminated intravascular coagulation, or other inflammatory or prothrombotic condition.

59. A method of treatment of a medical condition by administering to a patient in need a TM construct of the invention or conjugate thereof.

60. The method of claim 59 wherein the condition relates to a microvascular or macrovascular blood clot, stroke, heart attack, disseminated intravascular coagulation, or other inflammatory or prothrombotic condition.



Scheme A

FIG. 1

FIG. 2

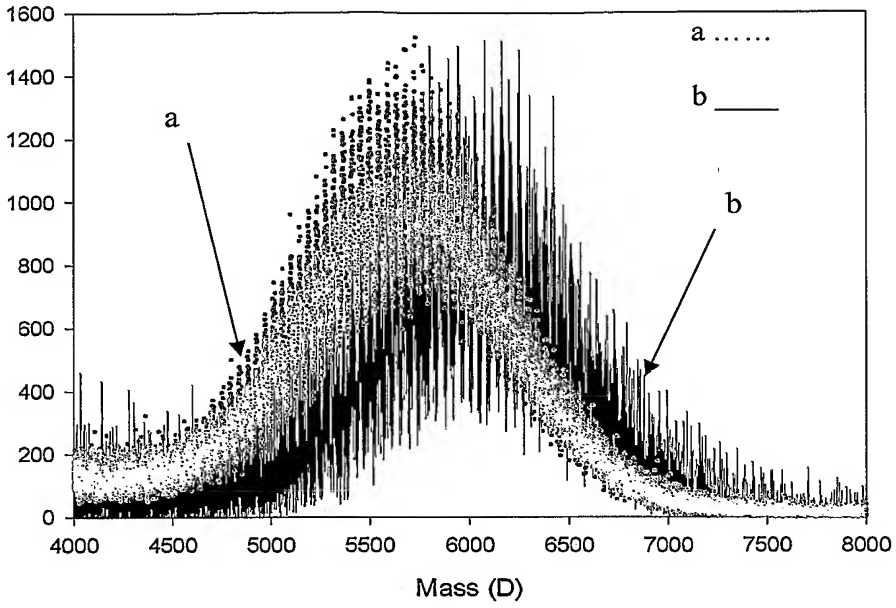


FIG. 3 (in color)

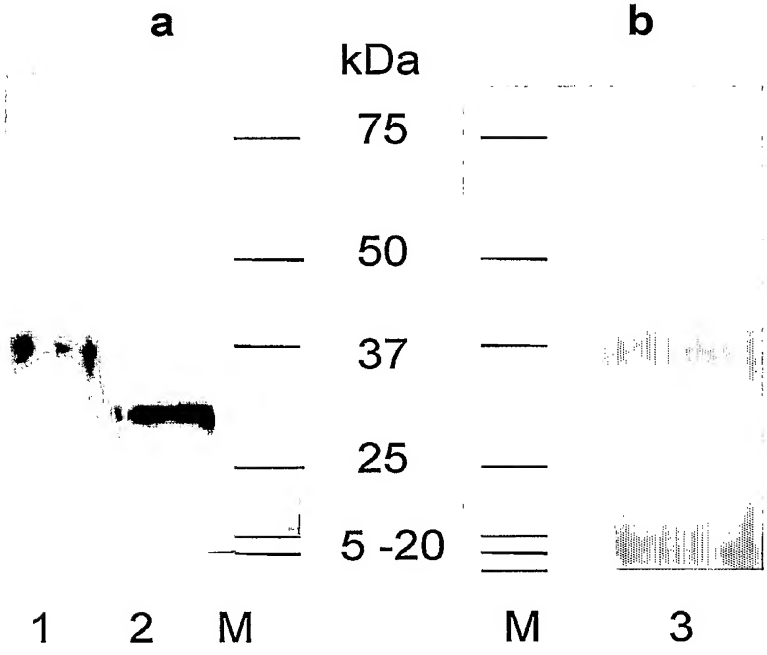
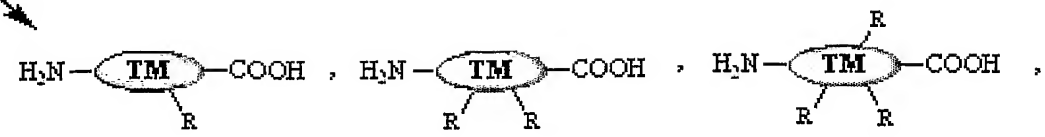


FIG. 4

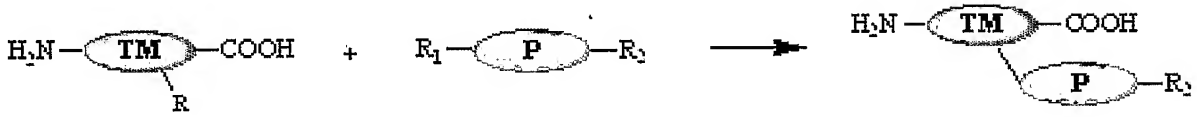
Genetically Directed Synthesis using non-natural amino-acid :



TM analogs containing NNAA

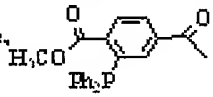
TM: Thrombomodulin analog capable of activating protein C
NH_2 : Amino group at the N-terminal of TM
COOH: Carboxylic acid group C-terminal of TM
NNAA: Non-Natural Amino Acid
R: Functional group of the non-natural amino acid (N_3, alkyl, diene, ...)

Example of a Conjugation Reaction:



TM analog containing one NNAA Linear polymer TM conjugate

P: Linear or branched natural or synthetic polymers such as PEG, oligosaccharides, ...

R_1 : Alkyne, diene,  , ...

R_2 : - Functional group for anchoring onto surface : alkyne, diene, biotin, ...

- Anti-inflammatory/anti-thrombotic groups such as heparin, sialic acid Lewis X, ...

FIG. 5

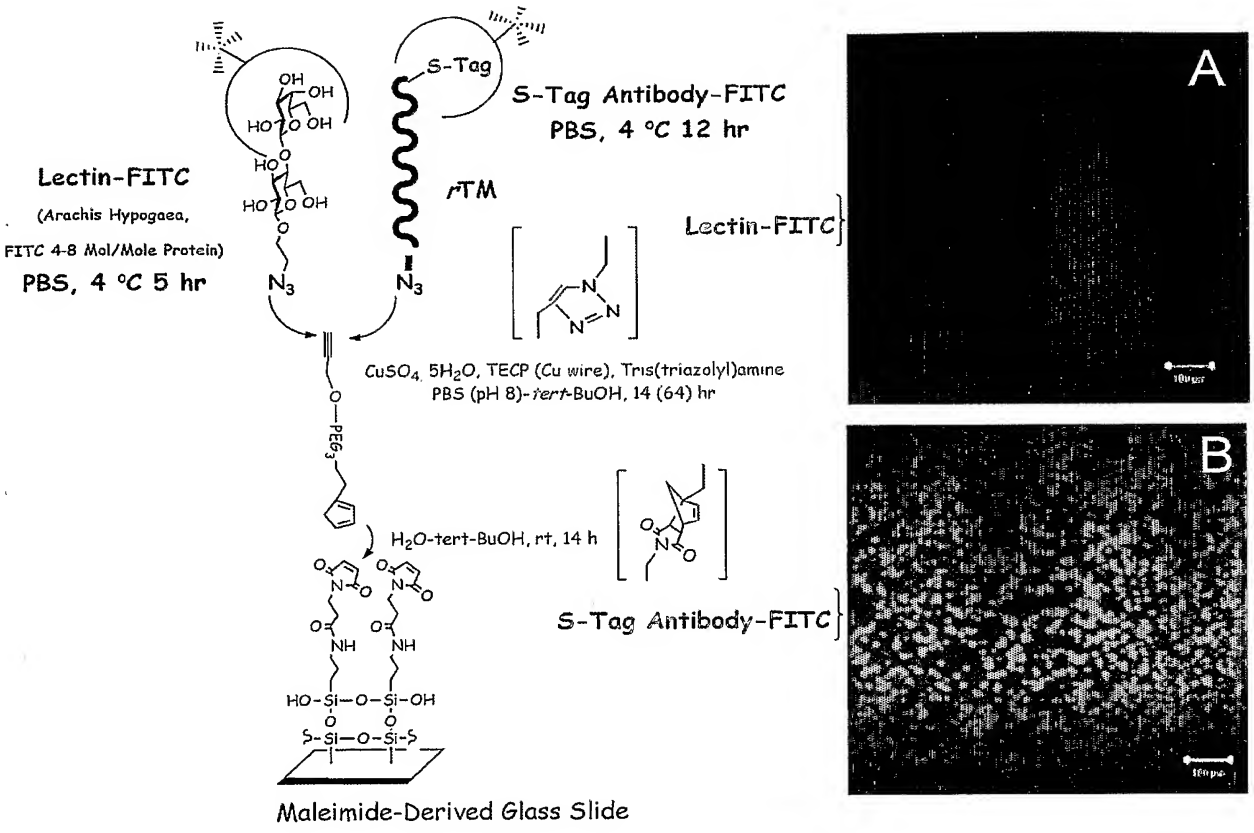


FIG. 6 (in color)

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APPLICANT: Emory University
CHAIKOF, Elliot L.
CAZALIS, Chrystelle S.
HALLER, Carolyn A.

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